The Use of Preserved Hematopoietic Tissues for Treatment of Mice Lethally Irradiated with Gamma-Rays under High Dose Rate. (I) : Effect of Preserved Isologous Bone Marrow (Special Issue on Physical, Chemical and Biological Effects of Gamma Radiation, VI)

AUTHOR(S):

Hama, Masaharu

The Use of Preserved Hematopoietic Tissues for Treatment of Mice Lethally Irradiated with Gamma-Rays under High Dose Rate. (I)

Effect of Preserved Isologous Bone Marrow

Masaharu HAMA*

The First Division, Department of Internal Medicine,
Faculty of Medicine, Kyoto University

Received January 30, 1965

Mice were irradiated lethally with gamma-rays under high dose rate and were treated with preserved isologous bone marrow.

The isologous bone marrow cells were suspended in 15% V/V glycerol-Tyrode's solution at −80°C as long as 450 days. As to the survival rate at 30 days after irradiation, the isologous bone marrow within 360 days preservation protected well the lethally gamma-irradiated mice as well as the fresh isologous bone marrow transplantation did, as far as 5 million of nucleated cells were injected.

INTRODUCTION

In acute radiation death, it is well known that three different mechanisms[1,2] of death resulting from the injuries of the central nervous system, intestinal tract or hematopoietic system must be considered separately. Radiation death from the central nervous system [3,4,5] occurs within minutes or hours after exposure when animals are exposed to the supralethal radiation dose (kiloroentgen). Intestinal death occurs 3-5 days after exposure, associated with sever and continuous diarrhoea.[6,7] Damage to the intestinal mucosa is the major cause of these symptoms. Apparently the denuded mucosa can not absorb enough water and electrolytes to prevent dehydration, salt loss, and other changes that are the immediate cause of death.

Radiation death from the hematopoietic system is one of the most important causes of death after total-body irradiation. Destruction of bone marrow is the most critical event in terms of nearly total loss of hematopoietic system and subsequently, extremely small numbers of granulocytes and platelets in peripheral blood are followed by bacterial invasion [8] and hemorrhage [9]. Bacterial infection and hemorrhage are the immediate causes of acute radiation death. The anemia resulting from the bone marrow failure is little significance in acute radiation death.

In 1951, Lorenz and coworkers[10] reported the first successful experiments in which the intravenous or intraperitoneal injection of isologous bone marrow protected well the total-body lethally irradiated mice from hematopoietic death.

* 浜　　将治

The long term preservation of bone marrow cells is essentially necessary for the clinical application of bone marrow transplantation. In 1949, Polge, Smith, and Parkers[11] reported the successful preservation under freezing and thawing of bovine spermatozoa using glycerine as a protective medium. And then many workers reported the transplantation of the bone marrow cells preserved at low temperature in use of glycerol as a protective could improve the survival of lethally irradiated mice. In 1959, Lovelock and Bishop[12] showed that dimethyl -sulphoxide protects living cells against freezing injury in a fashion very similar to that of glycerol, and M. J. Ashwood-Smith[13] used dimethyl-sulphoxide to protect mouse bone marrow against the damaging effects of freezing to and thawing from low temperature.

The following experiments were undertaken to ascertain the method of freezing and thawing for the preservation of the hematopoietic cells and to observe the protective effect of the isologous bone marrow cells which were preserved at −80°C as long as 450 days upon the lethally irradiated mice.

MATERIALS AND METHODS

Mice. Dd/s strain mice supplied from the Kyoto University Animals Center were used as the recipients and isologous bone marrow (IBM) donors. They were 8 to 9 week old female mice, weighing approximately 20-25g at the experiments. After irradiation and treatment, 8 to 10 mice were kept in wooden boxes measuring 15x21x30cm, and were fed wheat, dried fish (each other day) and were given tap water ad libitum.

Bone marrow cell suspensions. The donor mice were sacrificed by neck fracture and bilateral tibias and femurs were removed aseptically.[14] Both ends of the bones were cut, a small needle (size 1/5, used usually for tuberculin tests) was inserted into one end, and the bone marrow was flushed directly from the bilateral femurs and tibias with the cold sterile Tyrode's solution or TC199 (Difico) solution and bone marrow cells in it were washed out. Glycerol was added to Tyrode's solution so as to make the final concentration of 15% (V/V) [15~23] and dimethyl-sulphoxide (DMSO) was added to TC199 solution so as to make the final concentration of 10% (V/V).[12,13,24~27] There were suspended 20×10^6 nucleated bone marrow cells in 1.2cc of 15% glycerol-Tyrode's solution or 10% DMSO-TC199 solution. An ampoule containing 3.6cc of the bone marrow suspensions was sealed with flame. Each recipient, except the mice in the control group, was given 5×10^6 nucleated bone marrow cells via the tail vein within 4 to 6 hours after irradiation. The mice in control were lethally irradiated without any further treatment or were injected intravenously 0.5ml of Tyrode's solution after irradiation. There were contained 5×10^6 nucleated bone marrow cells in 0.3ml of the bone marrow cells souspensin and glycerol or DMSO was not removed when the suspension was used. However, the suspension was diluted with 0.3ml of Tyrode's solution when it was used. So that the final concentration of the compound injected was thus one-half that used for freezing.

Freezing and thawing. Slow[21,23,28~30] freezing was carried out so that the temperature was lowered 1-2°C/min. from 0°C until −25°C and then lowered to

−80°C at a rate not exceeding 10°C/min. The ampoules were placed in a metal box containing ice pieces. The ampoules were removed from the metal box and then placed into a plastic box which contained about 200ml of ethanol and it was cooled to 0°C by dry ice. About 2-3g of pieces of dry ice cooled the bath of the plastic box at a rate 1°C/min.

The second step of slow freezing was achieved by adding dry ice pieces into the plastic box cooled by the first step. As coolant solid CO_2 was used and about 10g dry ice cooled the plastic box bath at a rate less than 10°C/min. from −25°C to −80°. When −80°C was reached, the ampoules were rapidly transferred to another Dewar-flask containing a −80°C ethanol-frozen CO_2 mixture and stored in it.

Rapid freezing. When rapid freezing was necessary, the ampoules were rapidly placed in the Dewar-flask containing a −80°C ethanol-frozen CO_2 mixture.

Fast thawing. The ampoules were removed from the Dewar-flask and the contents were thawed in a 37°C water bath and in one minute were completely liquefied by stirring.

Slow thawing. The ampoules were removed from the Dewar-flask and placed in room temperature or in air at 4°C until the frozen contents were completely liquefied.

Cell viability. In these experiments eosin staining method[31,32] was used and the dye in minute quantities was added to the cell suspension and the exclusion or incorporation of the dye by the cells was observed microscopically. The ratio of cells stained or not against the counted cells was determined by direct counting. But the exposure of the cell suspension to the dye solution for a period longer than 5 minutes resulted in a substantial reduction of dye-excluding cells. This eosin staining test was used as a screenig test. Eosin-uptake was measured on many of the bone marrow suspensions both before and after freezing. In every case one drop of 1:500 eosin in Tyrode's solution was added directly to one drop of the bone marrow suspensions on the slide glass. And a cover slip was placed over the mixtured suspension. Stained cells were suspected to be dead and the percentage of nonviable bone marrow cells (stained cells) in counted total bone marrow cells was described.

Irradiation. A Co^{60} gamma-irradiation facility which belongs to the Institute for Chemical Research of Kyoto University was used in the present experiments. This facility is described in detail elsewhere.[33,34] Four to five mice were placed in a 10×10×10cm. paper box which was then placed at the center of an aluminum container. The container with the box was moved in and out of the place of irradiation (A or B in Fig. 1) as quickly as possible in order for the mice not to be exposed to an extra dose of gamma-rays before and after receiving a certain dose of irradiation. Mice placed at A can be expected to be irradiated under single, total-body dose, because gamma-rays come from practically all directions. Those placed at B, however, can not be expected to be so, because gamma-rays come from one side and the distance from the cylindrical array to each animal in the box varies a little.

All recipients were exposed to a single LD_{99}/14-day, LD_{100}/21-day dose of

total-body irradiation of 900r which was obtained for 27 sec. in October, 1962, and for 32 sec. in October, 1964.

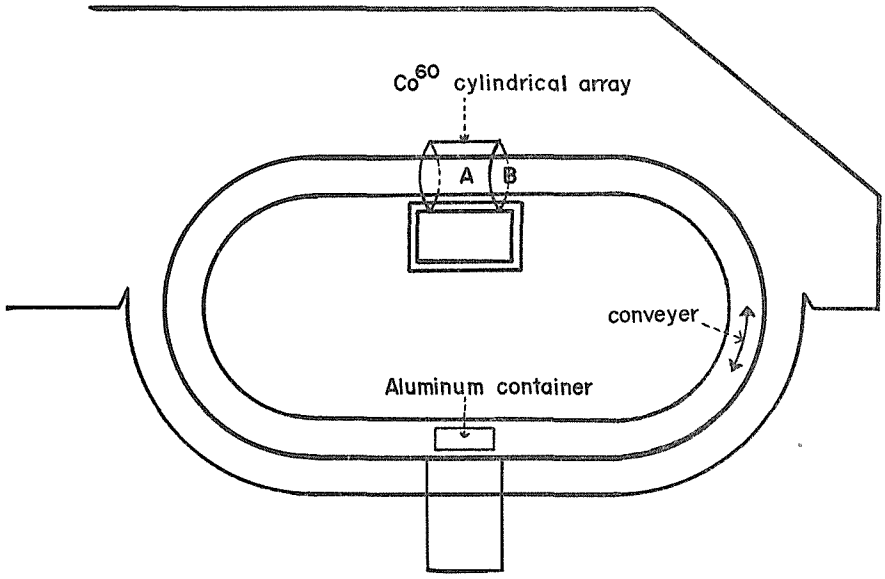


Fig 1. Co^{60} γ-ray irradiation facility.

RESULTS

1. Some Observations on the Basic Method of Preservation of Mouse Bone Marrow

Experiment 1. This experiment was carried out to protect the lethally irradiated mice by IBM cells which were suspended in Tyrode's solution and preserved in a refrigerator (0-4°C) for short term. Table 1 shows the survival rates of

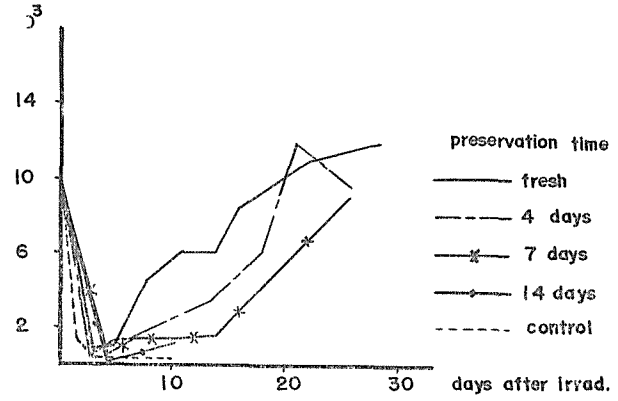
Table 1. Survival of gamma-irradiated mice treated with isologous bone marrow preserved at 0-4°C for 4, 7 and 14 days.

Preservation period	No. of experiment	Percent staining with eosin (%)	Number of survival / Number of irradiated (at days)				% Survival (at days)			
			14	30	60	90	14	30	60	90
Control	44	—	2/162	0/162	—	—	1	0	—	—
Fresh	2	9	14/19	13/19	13/19	13/19	74	68	68	68
4 days	1	64	4/8	3/8	3/8	3/8	50	37	37	37
7 days	1	73	6/8	4/8	3/8	3/8	75	50	37	37
14 days	1	97	0/8	—	—	—	0	—	—	—

The number of nucleated cells injected was 5 million. They were suspended in Tyrode's solution and presesrved at 0-4°C.

Control means the group of gamma-irradiated mice treated with Tyrode's solution or nothing.

Fresh means the group of gamma-irradiated mice treated with isologous bone marrow.



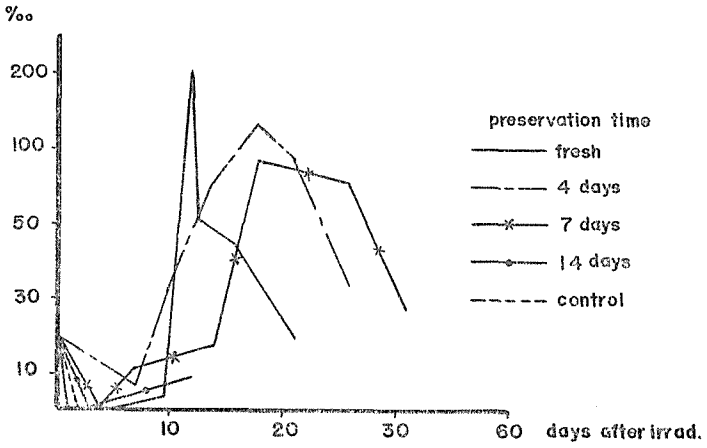


Fig. 2. Leucocyte count in gamma-irradiated mice treated with preserved isologous bone marrow at 0-4°C.
The number of nucleated cells injected was 5 million.
Preservation was carried out in Tyrode's solution for 4, 7 and 14 days.
Fresh means the group of gamma-irradiated mice treated with fresh isologous bone marrow.
Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing.

Fig. 3. Reticulocyte count in gamma-irradiated mice treated with preserved isologous bone marrow at 0-4°C.
The number of nucleated cells injected was 5 million.
Preservation was carried out in Tyrode's solution for 4, 7 and 14 days.
Fresh means the group of gamma-irradiated mice treated with fresh isologous bone marrow.
Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing.

irradiated mice treated with the preserved IBM transplantation and Figures 2 and 3 show the changes of leucocyte count and reticulocyte count in peripheral blood. The results showed that the IBM preserved for 4 days at 0-4°C could not already protect the lethally irradiated mice as well as the fresh IBM, and the IBM stored for 14 days at 0-4°C could not protect the early acute death of

lethally irradiated mice. As compared to the fresh IBM transplantation, this experiment indicated that the viability of bone marrow cells which were preserved at 0-4°C could be restained within 3 days. The survival rates at 30 days were as follows: 68% in the fresh IBM transplantation, 37% in the 4 days preserved IBM treatment, 50% in the 7 days preserved IBM treatment, and 0% in the 14 days preserved IBM treatment, respectively.

Experiment 2. This experiment was made to protect the lethally irradiated mice with IBM cells which were stored for 2 hours at −80°C without the protective additives. The IBM cells were suspended only in Tyrode's solution and were frozen and thawed rapidly. The percentage of eosin-staining cells in all counted cells was 100% and the survival rate at 14 postexposure days was 0% (0/8). This result showed that the cell viability was lost due to the freezing and thawing without protectives during the storage for 2 hrs at −80°C.

Experiment 3. This experiment was carried out to find the number of the bone marrow cells which could protect half of the lethally irradiated mice. The bone marrow cells were suspended in Tyrode's solution. The survival rates at 30 days after irradiation were as follows: when the number of nucleated cells injected was 0.1×10^6, 0.5×10^6, 1×10^6, and 2×10^6, the survival rate was 0%, 50%, 50% and 50%, respectively. So that the number of the bone marrow cells require for effective protection was 0.5×10^6 or less.

Experiment 4. For the mouse bone marrow preservation was used 15% glycerol-Tyrode's solution or 10% DMSO-TC199. The methods of freezing and thawing were various combinations of slow or fast freezing and slow or fast thawing.

Table 2. Survival rate of gamma-irradiated mice treated with preserved IBM.

Method of freezing, thawing	No. of experiment	Percent staining with eosin (%)	Number of survival (at days) / Number of irradiated				% Survival (at days)			
			14	30	60	90	14	30	60	90
Control	44	—	2/162	0/162	—	—	1	0	—	—
Fresh	2	9	14/19	13/19	13/19	13/19	74	68	68	68
↙↗ G	1	41	3/8	2/8	2/8	2/8	37	25	25	25
↙↗ D	1	23	6/8	5/8	5/8	5/8	75	62	62	62
↙↑ G	1	14	8/8	7/8	7/8	7/8	100	87	87	87
↙↑ D	1	23	6/8	6/8	6/8	6/8	75	75	75	75
↓↗ G	1	62	4/8	3/8	3/8	3/8	50	37	37	37
↓↗ D	1	49	4/8	3/8	3/8	3/8	50	37	37	37
↓↑ G	1	92	2/8	2/8	2/8	2/8	25	25	25	25
↓↑ D	1	90	3/8	3/8	3/8	3/8	37	37	37	37

The number of nucleated cells injected was 5 million. They was preserved for 2 hours at −80°C.

G means that IBM was suspended in 15% glycerol-Tyrode's solution.

D means that IBM was suspended in 10% DMSO-TC199.

Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing. Fresh means the group of gamma-irradiated mice treated with fresh IBM.

↙ means slow freezing of the bone marrow cells.

↓ means fast freezing of the bone marrow cells.

↑ means fast thawing frozen bone marrow.

↗ means slow thawing frozen bone marrow.

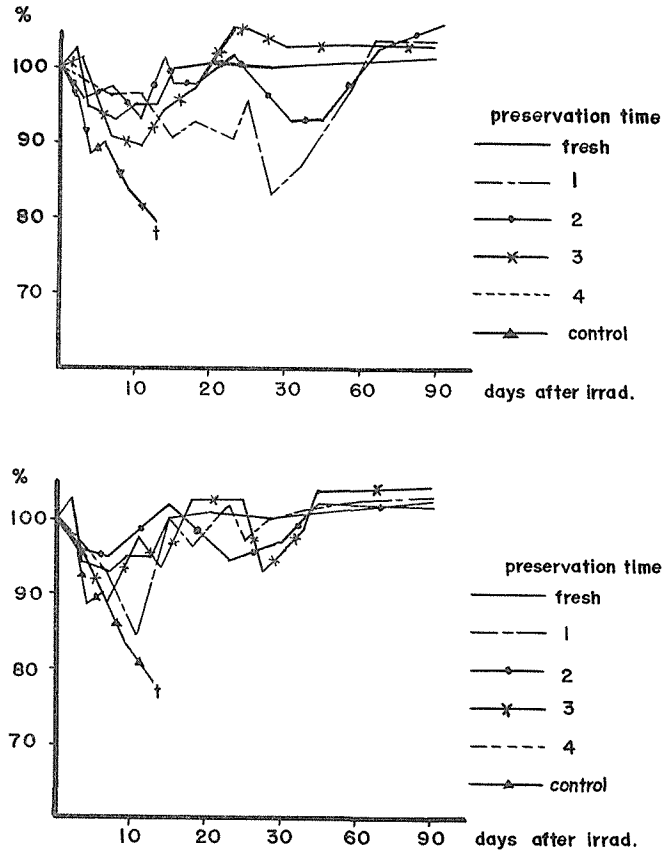


Fig. 4. Body weight changes in mice irradiated lethally and treated with preserved isologous bone marrow for 2 hours at -80°C. The number of nucleated cells injected was 5 million.
Control means the group of gamma-irradiated mice treated with only Tyrode's solution.
Fresh means the group of gamma-irradiated mice treated with fresh isologous bone marrow.
The upper figure shows body weight changes in mice irradiated lethally and treated with IBM suspended in 15% glycerol-Tyrode's solution.
The lower figure shows body weight changes in mice irradiated lethally and treated with IBM suspended in 10% DMSO-TC199.
1 means body weight changes in mice treated whih IBM which was slow frozen, stored and used after slow thawing.
2 means body weight changes in mice treated with IBM which was slow frozen, stored and used after fast thawing.
3 means body weight changes in mice treated with IBM which was fast frozen, stored and used after slow thawing.
4 means body weight changes in mice treated with IBM which was fast frozen, stored and used after fast thawing.

The survival rate of mice treated with fresh IBM was 74% at 14 days, 68% 30 days, 68% at 60 days and 68% at 90 days after irradiation, while, the survival rate of control group in which the lethally irradiated mice were treated only

with Tyrode's solution without any cell suspension was 1% at 14 days and 0% at 21 postexposure days. The bone marrow nucleated cells were suspended in 15% glycerol-Tyrode's solution. The survival rates were shown in Table 2, and the changes of body weight were shown in Fig. 4. The survival rate of mice treated with IBM in 15% glycerol-Tyrode's solution by slow freezing and slow thawing was 37% at 14 days and 25% at 30 days after irradiation. The survival rate of mice treated with preserved IBM in 10% DMSO-TC199 by the same method was 75% at 14 days and 62% at 30 days after irradiation.

The survival rate of mice treated with preserved IBM in 15% glycerol-Tyrode's solution by slow freezing and fast thawing was 87% at 30 days after irradiation. The survival rate of mice treated with IBM in 10% DMSO-TC199 by the same method was 75% at 30 days after irradiation. The survival rate of mice treated with preserved IBM in 15% glycerol-Tyrode's solution or 10% DMSO-TC199 by fast freezing and slow thawing was 37% and 37% at 30 days after irradiation, respectively. The survival rate of mice treated with preserved IBM in 15% glycerol-Tyrode's solution or 10% DMSO-TC199 by fast freezing and fast thawing was 25% and 37%, respectively.

The best survival rate was obtained by the method of slow freezing and fast thawing in 15% glycerol-Tyrode's solution and 10% DMSO-TC199. In the case of 10% DMSO-TC199, however, the good survival rate was obtained by the method of fast freezing and fast thawing. In order to freeze the mouse bone marrow nucleated cells for low temperature storage, the method of slow freezing and fast thawing is the fundamental procedure.

2. Observations on Long-term Preservation of Mouse Bone marrow at −80°C

This experiment was carried out to protect the lethally irradiated mice with the IBM cells which were preserved as long as 450 days at −80°C. The mouse bone marrow cells were suspended in 15% glycerol-Tyrode's solution and were stored at −80°C by slow freezing and used after thawing.

1) Survival rate. The survival rates in thirteen experiments are shown in Table 3 and Fig. 5. Observation period was 90 days after irradiation and the survival rate of mice treated with fresh IBM was 74% at 14 days, 68% at 60 days after irradiation, and 68% at 90 days after irradiation. In 150 days preserved IBM transplantation, the survival rate was 71% at 30 days, and 42% at 90 days after irradiation. In 270 days preserved IBM transplantaion, the survival rate was 71% at 30 days, and 71% at 90 days after irradiation. In one year (360 days) preserved IBM transplantation, the survival rate was 64% at 30 days, 47% at 60 days, and 29% at 90 days after irradiation. As for the survival rates at 30 days after irradiation, these were 40% to 84% and the survival rates at 90 days after irradiation, these were 29% to 76%. Consequently, the bone marrow cells were still effectvie after as long as one year preservation. As far as the survival rate at 90 days after irradiation is concerned, 360 days preserved IBM and 450 days preserved IBM transplantation could not protect the lethally irradiated mice as well as the fresh IBM transplantation.

Cell viability. As an index of cell viability the exclusion of dye (eosin) was

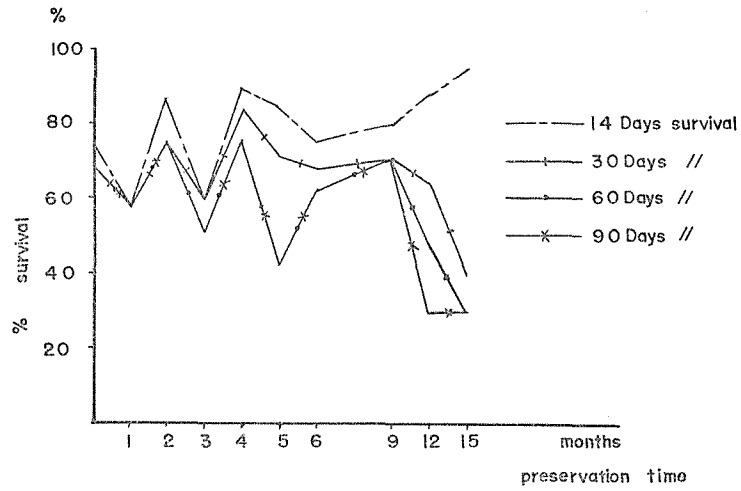


Fig. 5. Changes of percent survival in mice irradiated lethally and treated with preserved isologous bone marrow at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 30, 60, 90, 120, 150, 180, 270, 360 and 450 days.

Table 3. Survival rate of gamma-irradiated mice treated with preserved IBM.

Preservation period	No. of experiment	Percent staining with eosin (%)	Number of survival (at days) / Number of irradiated					% Survival			
			14	21	30	60	90	21	30	60	90
Control	44	—	2/162	0/162	—	—	—	0	—	—	—
Fresh	2	9	14/19	13/19	13/19	13/19	13/19	68	68	68	68
10 days	2	18	16/20	16/20	15/20	13/20	13/20	80	75	65	65
20 days	2	21	17/24	16/24	16/24	16/24	16/24	65	65	65	65
30 days	2	21	10/17	10/17	10/17	10/17	10/17	58	58	58	58
60 days	1	27	7/8	7/8	6/8	6/8	6/8	87	75	75	75
90 days	1	33	6/10	6/10	6/10	5/10	5/10	60	60	50	50
120 days	2	29	12/13	12/13	11/13	10/13	10/13	92	84	76	76
150 days	2	58	12/14	10/14	10/14	6/14	6/14	71	71	42	42
180 days	3	60	12/16	11/16	11/16	10/16	10/16	68	68	62	62
270 days	2	70	12/15	10/14	10/14	10/14	10/14	71	71	71	71
360 days	3	78	15/17	11/17	11/17	8/17	5/17	64	64	47	29
450 days	3	91	20/21	11/21	8/20	6/20	6/20	54	40	30	30

The number of nucleated cells injected was 5 million. They were preserved for 10 to 450 days at −80°C in 15% glycerol-Tyrode's solution.

Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing.

Fresh means the group of gamma-irradiated mice treated with fresh isologous bone marrow.

tested. In fresh IBM were eosin uptake cells 9%, and, generally speaking, long term preservation resulted in more eosin-uptake. In 450 days preserved IBM, eosin-uptake cells were 91%.

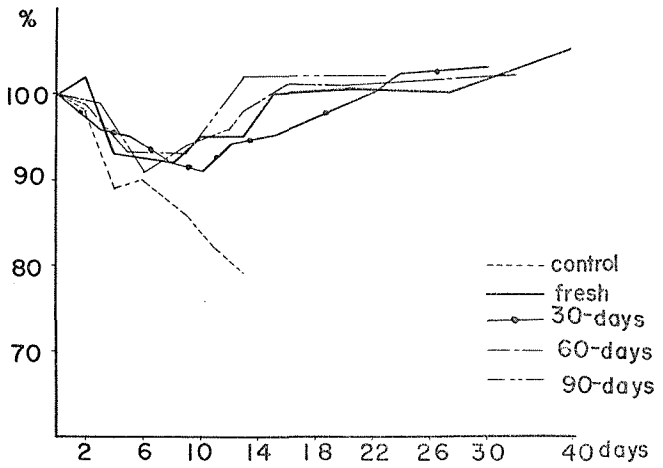


Fig. 6. Body weight changes in mice irradiated lethally and treated with preserved isologous bone marrow at -80°C. Preservation was carried out in 15% glycerol-Tyrode's solution for 30, 60 and 90 days. The number of nucleated cells injected was 5 million. Fresh means the group of gamma-irradiated mice treated with fresh IBM. Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing.

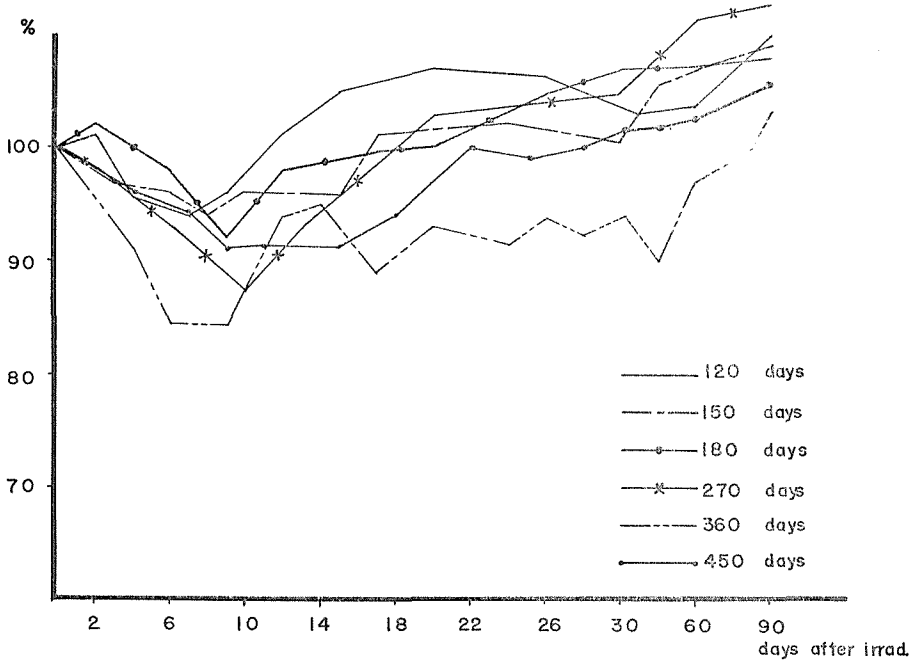


Fig. 7. Body weight changes in mice irradiated lethally and treated with preserved isologous bone marrow at -80°C. Preservation was carried out in 15% glycerol-Tyrode's solution for 120, 150, 180, 270, 360 and 450 days. The number of nucleated cells injected was 5 million.

2) **Body weight changes** Body weight changes are shown in Fig. 6 and Fig. 7. In the controls the weight loss continued until their death occurring within two weeks after irradiation.

Mice treated with preserved IBM showed a marked continuous weight loss for the eleven days following gamma-irradiation. However. the weight loss stopped at 5 to 11 days after irradiation and gradually increased thereafter. Except the mice treated with 270 days and 360 days preserved IBM, the body weight decreased to approximately 90% of the preirradiation level and it took 8 days in mice treated with fresh IBM, 10 days in mice treated with 30 days preserved IBM, 6 days in mice treated with 60 days preserved IBM, 9 days in mice treated with 90 days preserved IBM, 7 days in mice treated with 120 days preserved IBM, 8 days in mice treated with 150 days preserved IBM, 9 days in mice treated with 180 days preserved IBM, 10 days in mice treated with 270 days preserved IBM, 9 days in mice treated with 360 days preserved IBM, and 9 days in mice treated with 450 days preserved IBM. The weight loss of mice treated with 270 days preserved IBM decreased to approximately 87% of the preirradiation level and the weight loss of mice treated with 360 days preserved IBM decreased to about 84% of the pre-irradiation level.

Until the body weight recovered to the pre-irradiation level, it took 15 days in mice treated with fresh IBM, 22 days in mice treated with 30 days preserved IBM, 14 days in mice treated with 60 days preserved IBM, 15 days in mice treated with 90 days preserved IBM, 12 days in mice treated with 120 days preserved IBM, 17 days in mice treated with 150 days preserved IBM, 17 days in mice treated with 180 days preserved IBM, 18 days in mice treated with 270 days preserved IBM, 60 days in mice treated with 360 days preserved IBM, and 23 days in mice treated with 450 days preserved IBM. Namely, a retarded recovery of body weight

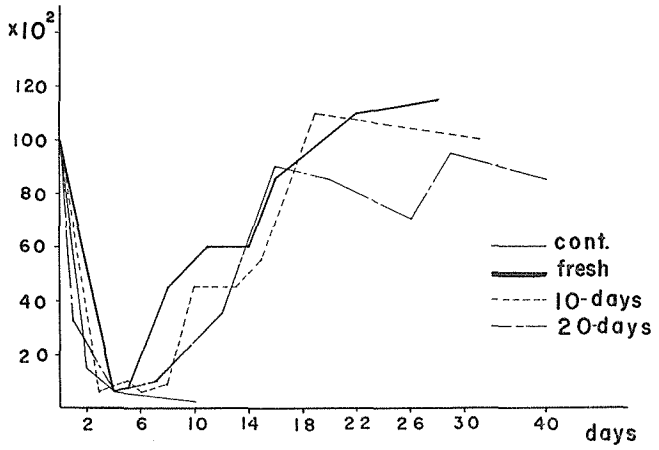


Fig. 8. Changes of leucocyte counts in mice irradiated lethally and treated with preserved isologous bone marrow at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 10 and 20 days. Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing. Fresh means the group of gamma-irradiated mice treated with fresh IBM.

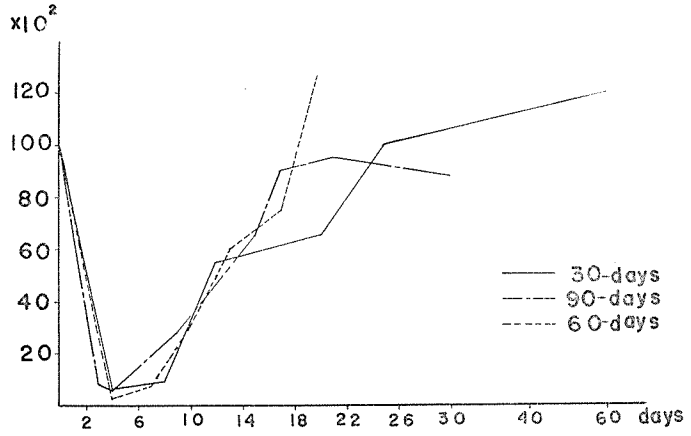


Fig. 9. Changes of leucocyte counts in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 30, 60 and 90 days.

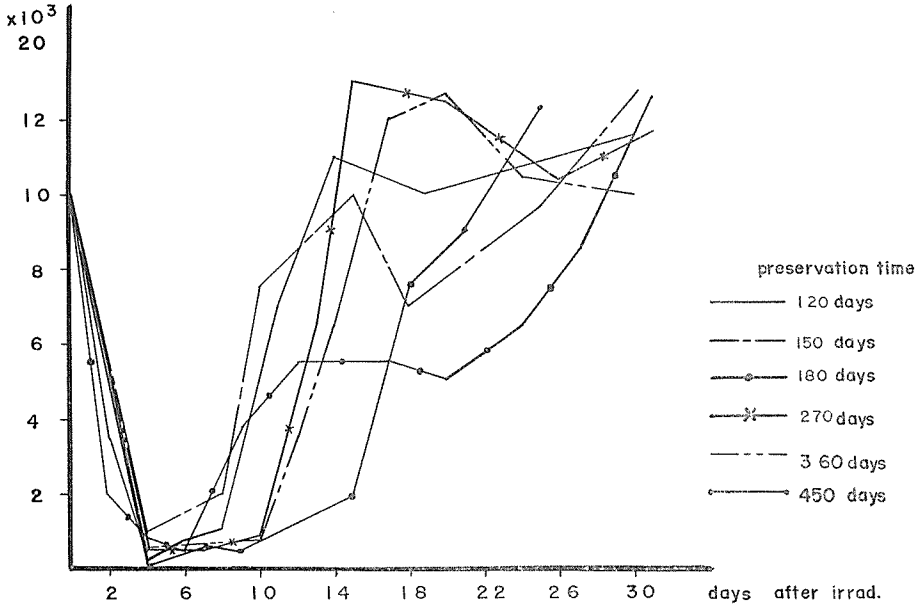


Fig. 10. Changes of leucocyte counts in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 120 150, 180, 270, 360 and 450 days.

for 2 to 8 days was found in mice treated with the preserved IBM, as compared to mice treated with the fresh IBM. But the recovery of body weight in mice with 360 days preserved IBM was retarded to 60 days after irradiation.

3) **Hematological findings.** a) Leucocyte count. The changes of leucocyte

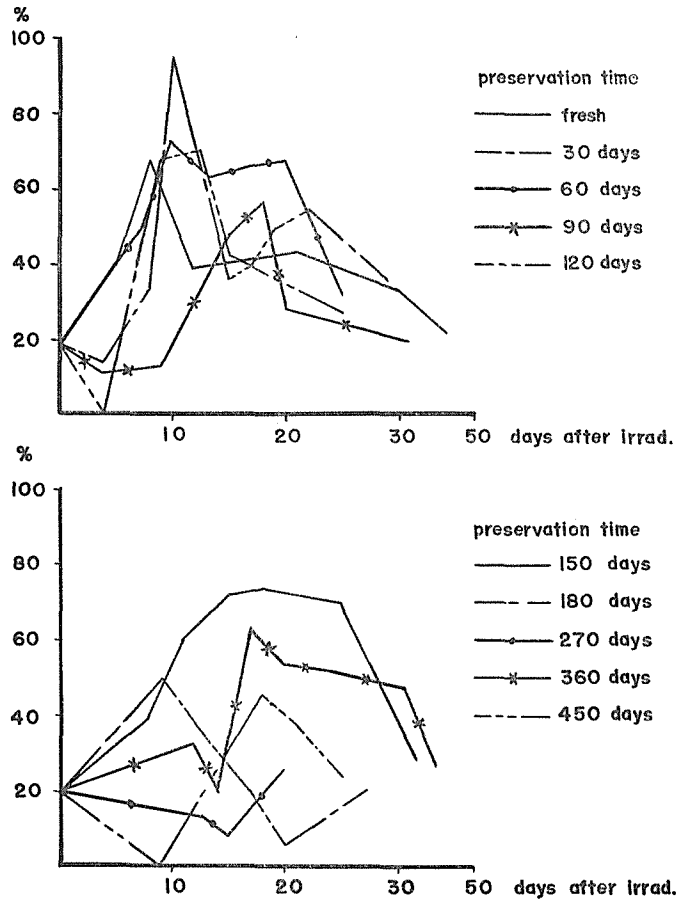


Fig. 11. Changes of the percentage of neutrophils in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 30, 60, 90, 120, 150, 180, 270, 360 and 450 days. Fresh means the group of gamma-irradiated mice treated with fresh isologous bone marrow.

count in peripheral blood were shown in Fig. 8, Fig. 9, and Fig. 10. Leucocyte count in peripheral blood was investigated every other day for 21 days after irradiation and weekly for 22 to 40 days after irradiation. The leucocyte count in both controls and treated mice showed a rapid decrease, 2000 to 4000 at 2 days after irradiation, and less than 500 at 4 days after irradiation. In controls there was no increase in leucocyte count up to the time of death. In treated mice the leucocyte count began to increase at 6 to 7 days after irradiation. At 10 days after irradiation, the leucocyte count was 6000 in fresh IBM transplantation, 4000 in 90 days preserved IBM, 1000 in 150 days preserved IBM transplantation, 1000 in 360 days or 450 days preserved IBM transplantation. Then it gradually or quickly approached normal levels. The time required for recovery to normal level was 19 days in fresh IBM, 25 days in 30 days preserved IBM transplantation, 21 days in 90 days preserved IBM

transplantation, 13 days in 120 days preserved IBM transplantation, 16 days in 150 days preserved IBM transplantation, 28 days in 180 days preserved IBM transplantation, 14 days in 270 days preserved IBM transplantation and 20 days in 450 days preserved IBM transplantation. Namely, the period for recovery in this experiment was not always later than that in fresh bone marrow transplantation. Neutrophils percentage. The changes of neutrophils percentage in peripheral blood were shown in Fig. 11. In the peripheral blood of mouse the difference between lymphocyte and monocyte was not clear, so that lymphocyte and monocyte were described as mononuclear cells. Neutrophils percentage is neutrophile leucocytes /neutrophile leucocytes+mononuclear cells. Peaks of neutrophils appeared between 9 to 25 days after irradiation and the peaks appeared later in long term preservation. The peaks recovered to normal level of pre-irradiation between 25 to 45 days after irradiation.

b) Erythrocyte count, reticulocyte count and hemoglobin content. The changes in the erythrocyte count following irradiation were shown in Fig. 12 and Fig. 13. The changes in erythrocyte count following irradiation were less than in leucocyte count. There was no apparent difference in erythrocyte count between the control mice and the treated groups 5 days after irradiation. The lowest level of the erythrocyte count was found at 2 to 16 days after irradiation and the count recovered to the level of pre-irradiation between 26 to 35 days after irradiation.

The changes in the reticulocyte count were shown in Fig. 14 and Fig. 15. The reticulocyte count in the controls decreased to zero by 2 days after irradiation. The reticulocyte count in the preserved IBM transplantations decreased to zero by 4 to 5 postexposure days. It increased up at 6 days. All groups of the mice treated with preserved IBM showed one reticulocyte crisis. The peak of reticulocyte count appeared at 12 days in mice treated with fresh IBM, at 19 days in mice

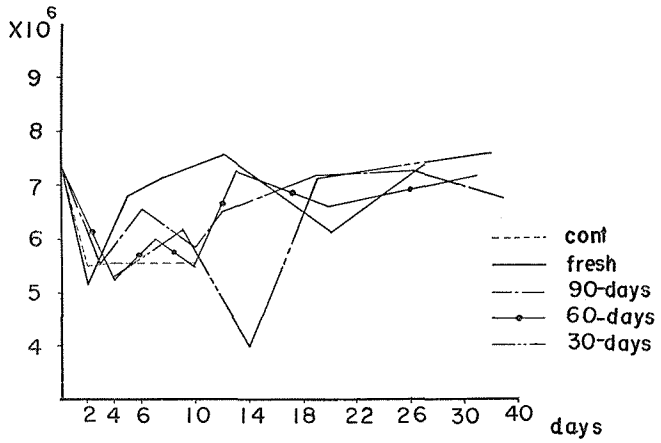


Fig. 12. Changes of erythrocyte counts in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 30, 60 and 90 days. Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing. Fresh means the group of mice irradiated lethally and treated with fresh IBM.

treated with 30 days preserved IBM, at 11 days in mice treated with 120 days preserved IBM, at 12 days in mice treated with 270 days preserved IBM, at 12

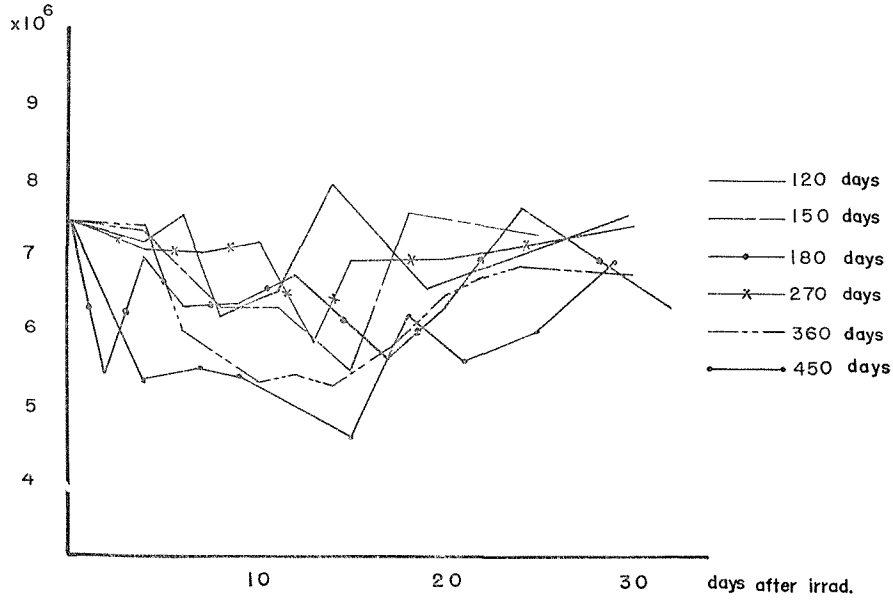


Fig. 13. Changes of erythrocyte counts in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 120, 150, 180, 270, 360 and 450 days.

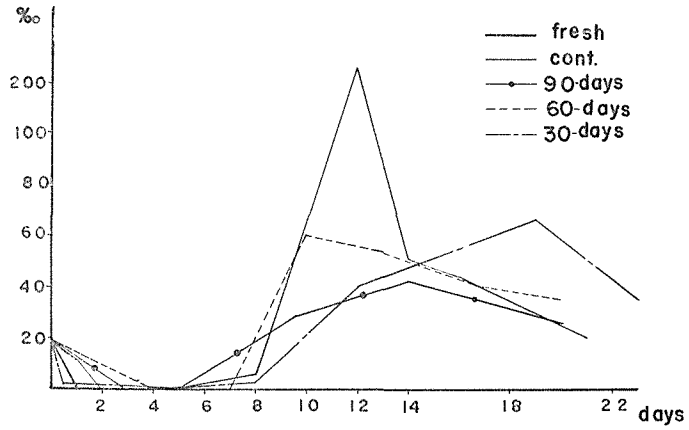


Fig. 14. Changes of reticulocyte counts in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 30, 60 and 90 days. Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing. Fresh means the group of gamma-irradiated mice treated with fresh IBM.

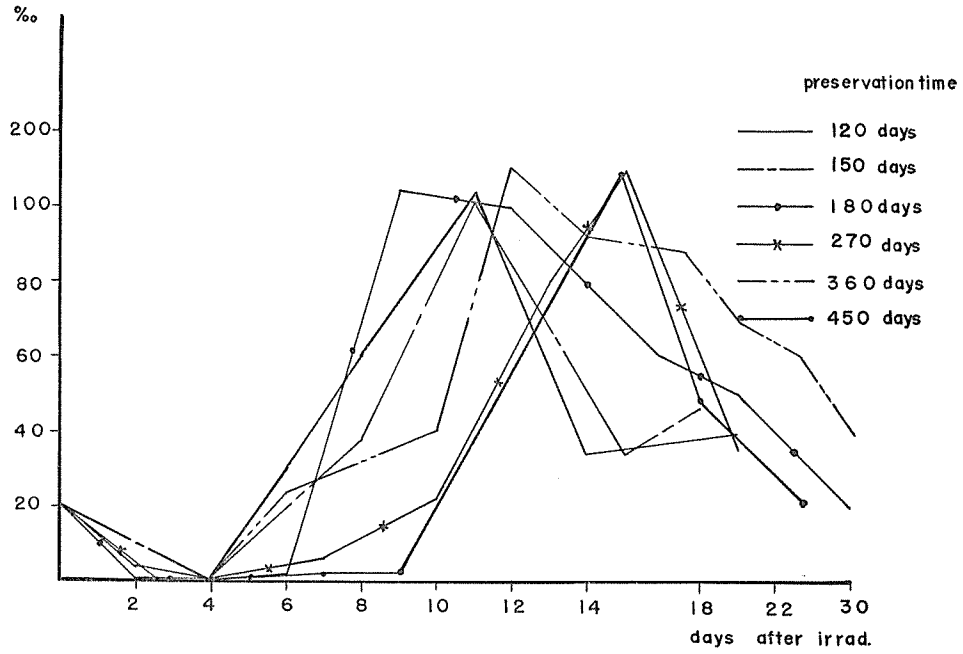


Fig. 15. Changes of reticulocyte counts in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 120, 150, 180, 270, 360 and 450 days.

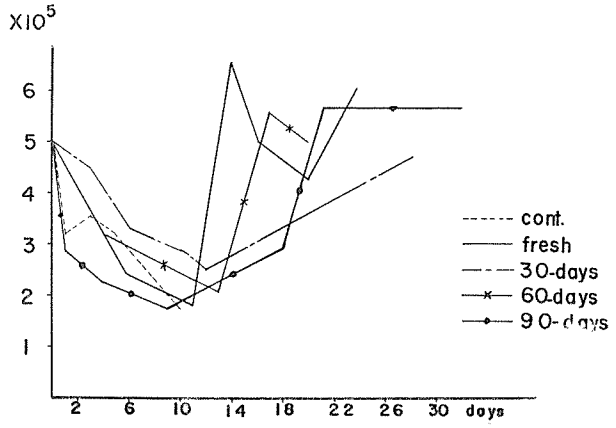


Fig. 16. Changes of platelet counts in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 30, 60 and 90 days. Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing. Fresh means the group of gamma-irradiated mice treated with fresh IBM.

days in mice treated with 360 days preserved IBM, and at 15 days in mice treated with 450 days preserved IBM. Namely, the peaks of reticulocyte count appeared

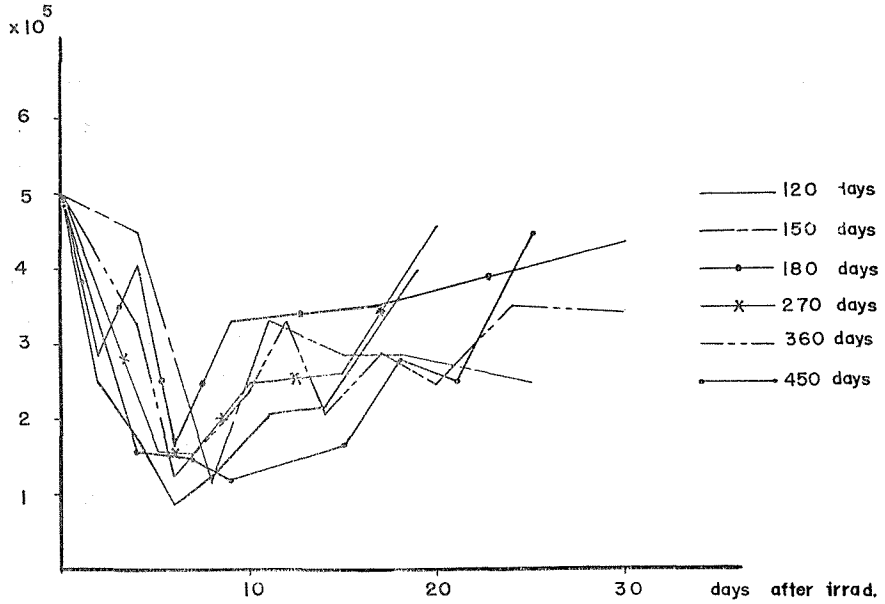


Fig. 17. Changes of platelet counts in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 120, 150, 180, 270, 360 and 450 days.

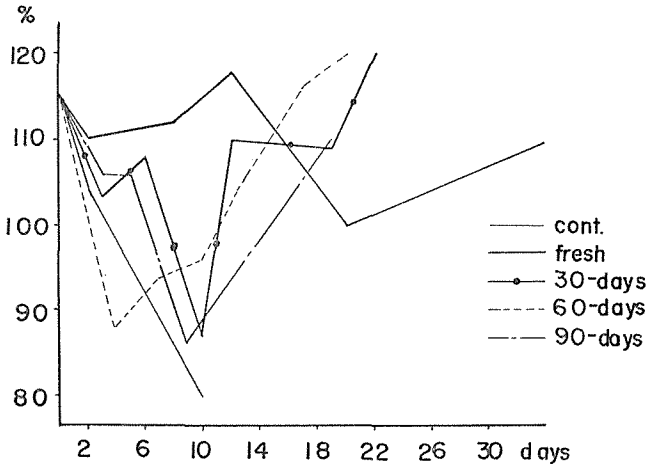


Fig. 18. Changes of hemoglobin contents in mice irradiated lethally and treated with preserved IBM at -80°C. The number of nucleated cells injected was 5 million. Preservation was carried out in 15% glycerol-Tyrode's solution for 30, 60 and, 90 days. Control means the group of gamma-irradiated mice treated with only Tyrode's solution or nothing. Fresh means the group of gamma-irradiated mice treated with fresh IBM.

between 11 to 19 days after irradiation. And the reticulocyte count recovered to the pre-irradiation value at about 22 to 30 days after irradiation.

The changes of platelet count in peripheral blood were shown in Fig. 16

and Fig. 17. The platelet count in controls rapidly decreased, showing temporary increase, to the level of 12×10^4. The platelet count in the treated mice with preserved IBM decreased to the level of 18 to 19×10^4 about 9 to 13 postexposure days and then increased rapidly or gradually up to the level of pre-irradiation at 16 to 30 days after irradiation. However, in this experiment there were found some cases which showed two phases in the changes of platelet count. The second lower level of the platelet count appeared at 19 to 21 days after irradiation in mice treated with fresh IBM, 150 days, 360 days, and 450 days preserved IBM.

The changes of hemoglobin content (Sahli) in peripheral blood were shown in Fig. 18 and Fig. 19. The hemoglobin content in controls rapidly decreased to the level of 80%. The hemoglobin content in the treated mice with preserved IBM decreased to the level of 85-100% at about 6 to 15 days after irradiation and then increased up to the level of pre-irradiation at 16 to 23 days after irradiation.

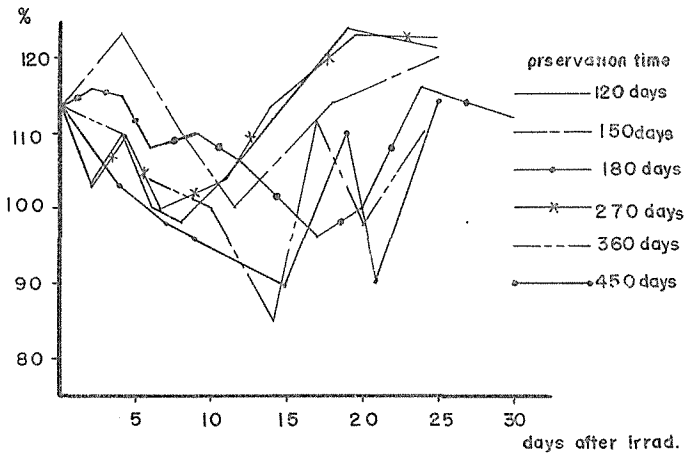


Fig. 19. Changes of hemoglobin contents in mice irradiated lethally and treated with preserved IBM at -80°C.
The number of nucleated cells injected was 5 million.
Preservation was carried out in 15% glycerol-Tyrode's solution for 120, 150, 180, 270, 360 and 450 days.

4) **Histological findings.** The examined tissues were femoral bone marrow, sternal bone marrow, thymus, mesenteric lymph node, brachial lymph node, and spleen.

Bone marrow. In both controls and mice treated with preserved IBM, only mature granulocytes and megakaryocytes remained in the bone marrow cavity 24 hours after irradiation. In untreated mice the aplastic bone marrow persisted until the death of the animals. However, the bone marrow of mice which were killed 10 days after irradiation showed a marked increase of reticular cells and scattered foci of hematopoiesis.

In mice treated with fresh bone marrow cells, early regeneration was initiated on the 4th day after irradiation. Scattered groups of regenerating young cells

Table 4. The times of appearance of early regeneration and complete recovery in the hematopoietic tissues in gamma-irradiated mice treated with preserved IBM.

Preservation period	Bone Marrow		Spleen red pulp		Spleen white pulp		Thymus		Lymph Node	
	eg.	cp.	eg.	cp.	eg.	cp.	eg.	cp.	eg.	cp.
Control	10	—	10	—	—	—	10	—	—	—
Fresh	4	14	5	21	14	21	5	10	14	21
30 days	4	21	5	21	12	35	5	10	10	30
60 days	4	18	4	18	11	28	4	8	11	28
90 days	4	21	8	21	15	32	6	12	12	28
120 days	4	14	4	21	10	21	6	21	10	25
150 days	4	18	7	18	18	35	7	18	18	35
180 days	4	20	7	23	23	35	7	18	23	35
270 days	4	21	7	21	21	40	6	15	20	40
360 days	5	20	7	20	20	30	5	20	20	35
450 days	7	21	6	14	18	28	5	8	18	28

The number of nucleated cells injected was 5 million. They were preserved for 30 to 450 days at −80°C in 15% glycerol-Tyrode's solution.

Control means the group of gamma-irradiated mice treated with only Tyrode's solution.

Fresh means the group of mice treated with fresh IBM.

eg. means early regeneration in the tissues in mice lethally irradiated and treated with fresh and preserved IBM.

cp. means complete recovery in the tissues in the treated mice.

The numbers in Table show the days after irradiation.

appeared. Collection of swollen reticular cells and undifferentiated immature cells (most of those are erythroblasts) existed in early regeneration. On the 5 to 6th day after irradiation the wasted bone marrow space was studded with foci or masses of immature blood forming cells. On the 8 th day the bone marrow showed cellularity of half of that in the normal bone marrow. On the 10 th day the cellularity increased to 2/3 of the normal bone marrow cellularity. On the 14 th day the initially wasted bone marrow recovered to a normal cellularity. In the mice treated with 30 days preserved IBM, early regeneration was initiated on the 4 th day and the complete recovery of bone marrow occurred on the 21 st day after irradiation.

In the mice treated with 60 days preserved IBM, the early regeneration was initiated on the 4th day and the normal, much better to say hyperplastic, recovery occurred on the 18 th day after irradiation. In the mice treated with 90 days preserved IBM, early regeneration was initiated on the 4 th day and the bone marrow recovered to almost half a normal cellularity on the 14th day and complete recovery occurred on the 21 st day after irradiation.

In the mice treated with 120 days preserved IBM, early regeneration was initiated on the 4 th day and complete recovery of the wasted bone marrow occurred on the 14 th day after irradiation.

In the mice treated with 150 days preserved IBM, early regeneration was initiated on the 4th day and complete recovery occurred on the 18 th day after irradiation.

In the mice treated with 180 days preserved IBM, early regeneration was init-

iated on the 4th day and complete recovery occurred on the 20th day after irradiation.

In the mice treated with 270 days preserved IBM, early regeneration was initiated on the 4th day and complete recovery occurred on the 21 st day after irradiation.

In the mice treated with 360 days preserved IBM, early regeneration was initiated on the 5th day and complete recovery occurred on the 20th day after irradiation.

In the mice treated with 450 days preserved IBM, early regeneration was initiated on the 7th day and complete recovery occurred on the 21 st day after irradiation.

Consequently, the early regenerations of the lethally irradiated mice in each group were initiated on the 4 th to 7th after the transplantions of the isologous bone marrow cells which had been preserved at -80°C for 10 to 450 days. Except the treatments by 360 days and 450 days preserved IBM, all early regenerations occurred on the 4 th day after the treatments.

The complete recovery in the bone marrow in each group occurred between the 14 th to 21 st day after treatments. It is interesting to note that the longer-term preserved bone marrow did not always delay the early regenerations and postpone complete recoveries.

Thymus

In 24 hours after irradiation, although almost complete disappearance of thymocytes from the cortex occurred, a small number of thymocytes remained in the medulla in all animals. However, in 4 days after irradiation, the thymus was completely wasted.

In the mice treated with the fresh bone marrow, early regeneration was initiated in the medulla on the 5 th day and the thymus recovered to normal cellularity on the 10 th day after irradiation.

In the mice treated with 30 days preserved IBM, early regeneration was initiated on the 5th day and complete recovery occurred on the 10 th day after irradiation.

In the mice treated with 90 days preserved IBM, early regeneration occurred on the 6th day and complete recovery occurred on the 12 th day after irradiation.

In the mice treated with 360 days preserved IBM, early regeneration occurred on the 5th day and complete recovery occurred on the 15 th day after irradiation.

In the mice treated with 450 days preserved IBM, early regeneration and complete recovery occurred on the 5 th day and on the 8 th day after irradiation, respectively.

Spleen

Red pulp. Hematopoiesis is noticed in the splenic red pulp throughout mouse lifespan. During the lst month after irradiation and bone marrow treatment, the spleen appears to be a blood-forming organ of prime importance. In the mice treated with the fresh bone marrow, the early regeneration was discernible on the 5 th day and the complete recovery occurred on the 21st day after irradiation.

In the mice treated with 60 days preserved IBM, early regeneration occurred

on the 4th day and complete recovery occurred on the 18th day.

In the mice treated with 270 days preserved IBM, early regeneration occurred on the 7th day and complete regeneration occurred on the 21st day after irradiation.

In the mice treated with 450 days preserved IBM, early regeneration occurred on the 6th day and complete recovery occurred on the 14th day after irradiation.

The first signs of regeneration were discernible on the 4 to 8 th day and complete recovery was disdernible on the 14th to 21st day after irradiation.

White pulp. The number of mature lymphocytes in the lymphatic tissues of spleen (white pulp) was greatly reduced 24 hours after irradiation. Reticular cells and pyknotic lymphocytes remained.

In the mice treated with fresh IBM, early regeneration in the white pulp was discernible on the 14th day and complete recovery occurred on the 21 st day after irradiation.

In the mice treated with 180 days preserved IBM, early regeneration was discernible on the 23th day and complete recovery occurred on the 25th day after irradiation.

In the mice treated with 450 days preserved IBM, early regeneration was discernible on the 18th day and complete recovery occurred on the 28th day after irradiation.

The first sign of early regeneration which was noticed as the appearance of small groups of lymphoblasts was discernible on the 10 th to 23 th day and the complete recovery occurred on the 21st to 40th day after irradiation.

Lymph node

In the mice treated with fresh IBM, early regeneration occurred on the 10th day and complete recovery occurred on the 21st day after irradiation.

In the mice treated with 360 days preserved IBM, early regeneration occurred on the 20th day and the complete recovery occurred on the 35th day after irradiation.

In the mice treated with 450 days preserved IBM, early regeneration occurred on the 18th day and complete recovery occurred on the 28th day after irradiation.

The first signs of early regeneration in the lymphatic tissues were discernible on the 10th to 23th day and complete recoveries occurred on the 21st to 40th day after irradiation.

The postirradiation days of early regenerations and complete recoveries in the hematopoietic tissues in the mice treated with fresh IBM and preserved IBM were shown in Table 4.

DISCUSSION

Since the pioneer work of Lorenz many workers reported the successful experiments in which the intravenous injection of isologous bone marrow protect well the total body lethally irradiated mice from hematopoietic death. Several workers[35-41] have shown by various methods that recovery is significant, if not entirely, due to recolonization of the normal cells injected in the host's radiation-damaged hematopoietic tissues. Evidence of Ford[35,36], Genogozian[42], and Zaalberg[43] indicated that the injected cells repopulate not only the bone marrow, but also the spleen, the thymus, and the lymphnodes.

Early investigators of tissue transplantation were restricted in the avalability of human tissues for clinical use. It would be very convenient for the clinical or experimental investigators to keep preserved human or animal tissues ready to be used. The advances of modern cryptobiology have resolved the difficulties of adequate freezing techniques, knowledge of effective protective agents, and statisfactory storage facilities.

The present experiments were carried out to protect the lethally irradiated mice with two combinations of bone marrow treatment and preservation. Under the condition of the experiments, a single total-body gamma-irradiation with 900r under high dose rate caused the death of all mice within 21 days after irradiation. The animals died of the bone marrow syndrome which was characterized by septicemia and hemorrhage.

1. The IBM which was preserved for 4 days at 0-4°C could not protect the lethally irradiated mice as well as the fresh bone marrow treatment. The IBM which was preserved for 14 days at 0-4°C could not protect the lethally irradiated mice. Urso[44] et al. reported that isologous bone marrow cells suspended in physiological saline solution stored for 1 or 2 days at 4°C gave the 30 day-survival similar to that obtained by fresh bone marrow. As compared with the fresh IBM transplantation, the viability of bone marrow cells which were preserved at 0-4°C could be restrained within 3 days.

The least number of isologous bone marrow cells with which a half of the lethally irradiated mice could survive was 5×10^6 or less. However, Uchino and Yamagishi[45] reported that one hundred thousand IBM cells gave approximately 45% 30 day survival. According to Urso and Congdon[46], the cell number of IBM was 0.6×10^6 or less.

To preserve the living cells or tissues for a long term, the first essential method is a reduction in the rate at which physical and chemical phenomena take place. This method was obtained by low temperatures, -80°C (ethanol and dry ice) or -196°C (liquid nitrogen). The dominant phemonena with lowering of temperature, is phase change, for an example, the freezing of water consists in the change of phase from liquid to crystalline solid. The formation of ice from water is one of the most important physical changes which take place when tissue is carried to low temperature. According to Meryman[29,30], and Mazur[47], it seems that death is associated with direct effects of ice crystal formation and not the indirect effects of high solute concentration. The intracellular ice formation seems to be the lethal factor. The cells can survive total extracellular ice formation.

The IBM cells which were preserved for 2 hours at -80°C without protectives could not protect the lethally irradiated mice. However, Ferrebee[16] reported that the bone marrow cells which were preserved for a few minutes at -80°C without protectives could protect the lethally irradiated mice. Meryman[29] reported that the living cells could be only protected from freezing injury, if the exposure to it had been brief, that is, a matter of minutes or seconds.

In 1949, Polge[12] and associates reported the first successful freezing of bovine semen with glycerol as protective medium. And then dimethyl-sulphoxide[13,14], and polyvinylpyrrolidons[26] were found to be protectives from freezing injury. The best protective compounds were found among either polyalcohols or monosaccharides. In

the bone marrow transplantation, no attempts were made to remove these compounds after thawing for use. To preserve the bone marrow cells, the best method consists in suspending the cells in 15% glycerol-solution[15,16,20-23,29] or in 10-15%[13,26] DMSO solution. The best method of freezing and thawing was slow freezing and fast thawing. To know the best method of freezing and thawing, many combinations of freezing and thawing were carried out. Slow freezing is the best method to protect the intracellular ice formation[20,21,23,29,30] of cells. Slow freezing was obtained by many methods. Barnes et[28] al. reported the apparatus of slow freezing. The first step of slow freezing with which the bone marrow cells must be cooled at a rate 1-2°C/min. is achieved by many methods. The ampules were immersed in an ice-alcohol mixture[48] and placed in a freezer maintained at -25°C until their contents were solidly frozen. Placing the ampules in a freezer at -20°C for 25 minutes[18] or 1/3 V/V salt and 2/3 V/V ice pieces could cool the contents of the ampules at the rate of 1-2°C/min. The second step of slow freezing which means cooling the contents from -25°C to -80°C at a rate not exceeding 10°C/min. was achieved by the use of solid CO_2 as coolant.

The IBM cells suspended in 15% glycerol-Tyrode's solution or in 10% DMSO-TC199 were used. The best 30 day-survival rates were obtained by the method of slow freezing and fast thawing in both suspensions.

2. Long-term preservation of isologous bone marrow cells. IBM cells suspended in 15% glycerol-Tyrode's solution were preserved for 10 to 450 days at -80°C by the method of slow freezing and fast thawing.

As for 30 days survival rates, the bone marrow cells which were preserved as long as 360 days could protect the lethally irradiated mice as well as the fresh IBM treatment. However, the survival rate of the mice treated with 450 days preserved IBM was 40% at 30 days as compared with 58 to 84% survival rates of the other treatments. As for 90 days survival, the bone marrow cells preserved as long as 270 days could protect the lethally irradiated mice as well as the fresh IBM treatment.

Cell viability. To test cell viability, there are many methods[22,27,31], such as enzymatic activity tests, cellular activity in tissue culture, mitotic figures, dye exclusion, and DNA synthesis. In these experiments eosin-uptake test was used, because an intact membrane of viable cells will presumably exclude such dyes as eosin. Eosin was usually prepared as a 0.2% Tyrode's solution. Generally speaking, the longer IBM cells were preserved, the more were there eosin-stained cells. The protective effect of bone marrow treatment itself was an evidence of cell viability.

The changes of body weight in the mice treated with preserved IBM were almost equal to the change of body weight in the mice treated with fresh IBM. However, the changes of body weight in the mice treated with 360 days preserved IBM or 450 days preserved IBM recovered to the preirradiation level later than those in the fresh IBM treatment.

The changes of hematological findings in the mice treated with preserved IBM were equal or later by 7 days than those in the fresh IBM treatment. The appearance of early regenerations in the bone marrow, the thymus, and the red pulp of spleen in the mice treated with preserved IBM were equal or 2 days later, as compared with that of the mice treated with fresh IBM. Complete recoveries

in them were equal or 10 days later than those in the fresh IBM treatment.

The appearance of early regenerations in the lymphatic tissues in the mice treated with preserved IBM was equal or 11 days later as compared with that in the fresh IBM treatment. Complete recoveries in them were equal or 20 days later than those in the fresh IBM treatment. It was expected that the longer the bone marrow cells were preserved, the later appeared the early regenerations and complete recoveries, but it was not necessarily so.

As for 30 day-survival rate, the survival rate of mice treated with fresh IBM was 68%, and the survival rate of mice treated with 270 days preserved IBM was 71%. The survival rate in the mice treated with 360 days preserved IBM was 64 %. Based on these results, the following conclusions were drawn: if it is assumed that the lethally irradiated mice could be protected from death with bone marrow consisting of erythropoietic component and leucopoietic component in a certain ratio, the effect of freezing injury to the two components was equal.

Bender[49] reported that the bone marrow cell suspended in 15% glycerol-solution which had been preserved for one year at -70°C could protect the lethally irradiated mice. However, this was the survival at 30 days.

As for 30 days survival rate, the bone marrow cells preserved for one year in 15% glycerol-Tyrode's solution, still retained the protective effect when the number of nucleated bone marrow cells injected was 5 million.

As for 90 days survival rate, the bone marrow cells preserved for 270 days in 15% glycerol-Tyrode's solution was still effective when the number of cells injected was 5 million.

ACKNOWLEDGMENTS

It is a great pleasure to thank Professor G. Wakisaka, M. D. and Dr. H. Uchino, M. D. and Dr. M. Yamagishi, and Dr. K. Adachi for their valuable suggestions and advices in carrying out the study here reported. Thanks are also extended to Mr. R. Katano, Institute for Chemical Research of Kyoto University, for his kindness in frequently operating the Co^{60} irradiation facility.

This work was supported by a grant-in-aid from the International Atomic Energy Agency 138/RB (1962-1964), to which thanks are due.

REFERENCES

(1) L. H. Smith, and C. C. Congdon, "Experimental treatment to acute whole-body radiation injury in mammals," Radiation Protection and Recovery (Edited by A. Hollaender), **7**, 242, Pergamon press, Oxford, 1960.
(2) E. P. Cronkite, and G. Brecher, *Ann. Rev. Med.*, **3**, 193 (1952).
(3) K. L. Krabbenhoff, *Am. J. Roetgenol. Radium Therapy and Nuclear Med.*, **73**, 850 (1955).
(4) H. B. Gerster, and S. P. Kent, *Radiation Research*, **6**, 626 (1957).
(5) H. L. Andrews, K. C. Brace, *Am. J. Physiol.*, **187**, 378 (1956).
(6) R. A. Conard, *Radiation Research*, **5**, 167 (1956).
(7) H. Quastler, *Radiation Research*, **4**, 303 (1956).
(8) C. P. Miller, C. W. Hammond, and M. Tompkins, *J. Lab. Clin. Med.*, **38**, 331 (1952).
(9) A. C. Upton, *Blood*, **10**, 1156 (1955).
(10) E. Lorenz, D. Uphoff, T. R. Reid, and E. Shelton, *J. Nat. Cancer Inst.*, **12**, 197 (1951).
(11) C. Polge, A. U. Smith, and A. S. Parkers, *Nature*, **164**, 666 (1949).
(12) J. E. Lovelock, and M. W. H. Bishop, *Nature*, **183**, 1394 (1959).

(13) M. J. Ashwood-Smith, *Nature*, **190**, 1204 (1961).
(14) M. Yamagishi, *This Bulletin*, **37**, 440 (1959).
(15) I. R. Schwartz, E. F. Repplinger, C. C. Congdon, and L. H. Tocantins, *J. Apply. Physiol.*, **11**, 22 (1957).
(16) J. W. Ferrebee, D. Billen, I. M. Urso, W. C. Lu, E. D. Thomas, and C. C. Congdon, *Blood*, **12**, 1096 (1957).
(17) H. E. Swim, R. F. Haff, and R. F. Parker, *Cancer Research*, **18**, 711 (1958).
(18) H. L. Lochte, J. W. Ferrebee, and E. D. Thomas, *J. Lab. & Clin. Med.*, **53**, 117 (1959).
(19) T. S. Hauschka, J. T. Mitchell, and D. J. Niederpruem, *Cancer Research*, **19**, 643 (1959).
(20) M. A. Bender, P. T. Tran, and L. H. Smith, *J. Apply. Physiol.*, **15**, 520 (1960).
(21) P. T. Tran, and M. A. Bender. *J. Apply. Physiol.*, **15**, 939 (1960).
(22) V. Richards, and M. Persidsky, *Surgery*, **50**, 288 (1961).
(23) V. P. Perry, *Federation Proc.*, **22**, 102 (1963).
(24) J. S. Porterfield, and M. J. Ashwood-Smith, *Nature*, **193**, 548 (1962).
(25) R. M. Dougherty, *Nature*, **193**, 550 (1962).
(26) M. Persidsky, and V. Richards, *Nature*, **197**, 1010 (1963).
(27) D. E. Pegg, *J. Apply. Phsiol.*, **19**, 123 (1964).
(28) D. W. H. Barnes, and J. F. Loutit, *J. Nat. Cancer Inst.*, **15**, 901 (1955).
(29) H. T. Meryman, *Ann. N. Y. Acad. Sci.*, **85**, 503 (1960).
(30) H. T. Meryman, *Federation Proc.*, **22**, 81 (1960).
(31) R. Schrek, *Am. J. Cancer*, **28**, 389 (1936).
(32) J. H. Hanks, and J. H. Wallace, *Proc. Soc. Exptl. Biol. & Med.*, **98**, 188 (1958).
(33) S. Okamoto, and Y. Nakayama, *This Bulletin*, **37**, 299 (1959).
(34) S. Shimizu, S. Tanaka, and Y. Nakayama, *This Bulletin*, **37**, 306 (1959).
(35) C. E. Ford, J. L. Hamerton, D. W. H. Barnes, and J. F. Loutit, *Nature*, **177**, 452 (1956).
(36) C. E. Ford, P. L. T. Ilbery, and J. F. Loutit, *J. Cellular Comp. Physiol.*, **50**, (Supp. 1), 109 (1957).
(37) P. C. Nowell, L. J. Cole, J. G. Habermeyer, and P. L. Roan, *Cancer Research*, **16**, 258 (1956),
(38) P. C. Nowell, L. J. Cole, P. L. Roan, and J. G. Habermeyer, *J. Nat. Cancer Inst.*, **18**, 127 (1957).
(39) O. Vos, J. A. G. Davids, W. W. H. Weyzen, and P. W. Bekkun, *Acta Phsiol. Neerl.*, 4, 482 (1956).
(40) T. Makinodan, *Proc. Soc. Exptl. Biol. & Med.*, **92**, 174 (1956).
(41) N. A. Mitchison, *Brit. J. Exptl. Path.*, **37**, 239 (1956).
(42) N. Genogozian, I. S. Urso, C. C. Congdon, A. D. Conger, and T. Makinodan, *Proc. Soc. Exptl. Biol. & Med.*, **96**, 714 (1957).
(43) O. B. Zaalberg, O. Vos, and D. W. Bekkum, *Nature*, **180**, 238 (1957).
(44) I. S. Urso, and C. C. Congdon, *J. Apply. Physiol.*, **10**, 314 (1957).
(45) H. Uchino, and M. Yamagishi, *Acta Haem. Jap.*, **24**, 656 (1961).
(46) P. Urso, and C. C. Congdon, *Blood*, **12**, 251 (1957).
(47) P. Mazure, *Blood*, **29**, 516 (1962).
(48) C. S. Stulberg, H. D. Soule, and L. Berman, *Pro. Soc. Exptl. Biol. &* Med., **98**, 428 (1958).
(49) M. A. Bender, and P. T. Tran, *Blood*, **29**, 520 (1962).

Explantation of Plates

Figs. 1-8: **Bone marrows**

Fig. 1. Bone marrow; 3 days after 900r gamma-irradiation.
The bone marrow is completely wasted. H-E stain ×100

Fig. 2. Bone marrow; 4 days after 900r gamma-irradiation and 60 days preserved IBM treatment. A group of immature bone marrow cells are seen to have appeared from the wasted bone marrow. H-E stain ×400

Fig. 3. Bone marrow; 4 days after 900r gamma-irradiation and 180 days preserved IBM treatment. A group of immature bone marrow cells are seen. This is early regeneration.

H-E stain × 400

Fig. 4. Bone marrow; 4 days after 900r gamma-irradiation and 450 days preserved IBM treatment. A group of immature bone marrow cells are seen. H-E stain ×400

Fig. 5. Bone marrow; 7 days after 900r gamma-irradiation and 270 days preserved IBM treatment. Marked regeneration is seen in the bone marrow. H-E stain × 400

Fig. 6. Bone marrow; 8 days after 900r gamma-irradiation and fresh IBM treatment. The cellularity is almost 2/3 of normal bone marrow. H-E stain × 400

Fig. 7. Bone marrow; 14 days after 900r gamma-irradiation and 60 days preserved IBM treatment. The cellularity is half a normal bone marrow. H-E stain × 100

Fig. 8. Bone marrow; 28 days after 900r gamma-irradiation and 450 days preserved IBM treatment. The cellularity is normal except for scattered areas of fatty change. H-E stain × 100

Figs. 9-17: **Spleens**

Fig. 9. Spleen; 4 days after 900r gamma-irradiation and fresh IBM treatment. The red pulp is completely wasted. H-E stain × 400

Fig. 10. Spleen; 8 days after 900r gamma-irradiation and fresh IBM treatment. Immature erythrocytic cells and myelocytic cells are seen in the red pulp. H-E stain × 400

Fig. 11. Spleen; 14 days after 900r gamma-irradiation and 360 days preserved IBM treatment. Marked regeneration in the red pulp is seen and the white pulp is wasted. H-E stain × 100

Fig. 12. Spleen; 10 days after 900r gamma-irradiation and 60 days preserved IBM treatment. Marked regeneration of myelocytic and erythrocytic cells are seen in the red pulp. H-E stain × 400

Fig. 13. Spleen; 7 days after 900r gamma-irradiation and 360 days preserved IBM treatment. The white pulp is completely wasted. H-E stain × 400

Fig. 14. Spleen; 16 days after 900r gamma-irradiation and fresh IBM treatment. Reticular cells and little lymphocytes are seen in the white pulp. H-E stain × 400

Fig. 15. Spleen; 21 days after 900r gamma-irradiation and fresh IBM treatment. H-E stain ×400

Fig. 16. Spleen; 30 days after 900r gamma-irradiation and fresh IBM treatment. A marked increase in number of lymphocytes in the white pulp. H-E stain × 400

Fig. 17. Spleen; 28 days after 900r gamma-irradiation and 450 days preserved IBM treatment. A marked increase in number of lymphocytes in the white pulp. H-E stain ×100

Figs. 18-20: **Thymus**

Fig. 18. Thymus; 4 days after 900r gamma-irradiation and 450 days preserved IBM treatment. The thymus is completely wasted. H-E stain × 400

Fig. 19. Thymus; 14 days after 900r gamma-irradiation and 360 days preserved IBM treatment. Marked regeneration in the thymic medulla. H-E stain × 100

Fig. 20. Thymus; 14 days after 900r gamma-irradiation and 450 days preserved IBM treatment. The cellularity is normal. H-E stain × 100

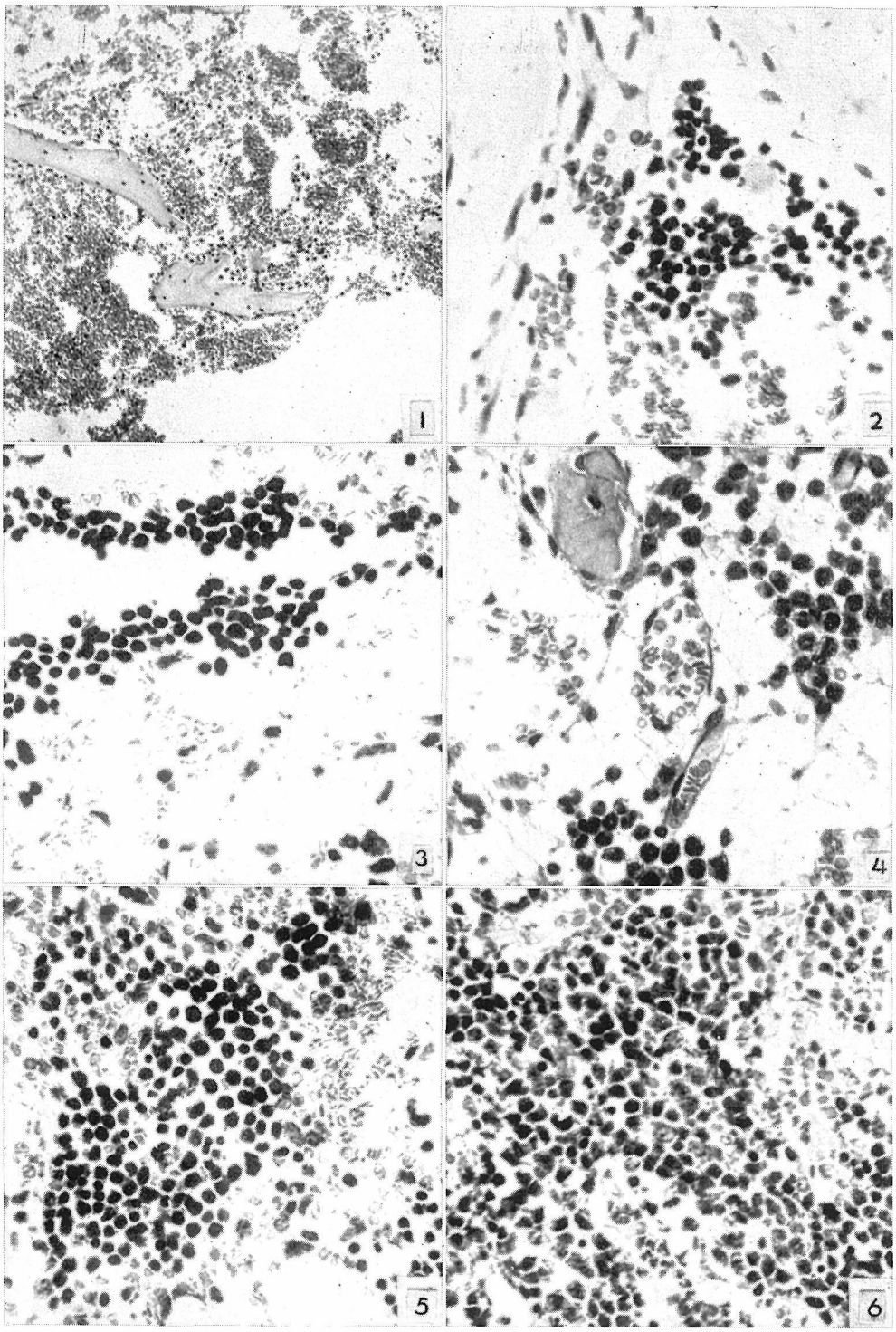
Figs. 21-24: **Lymphnodes**

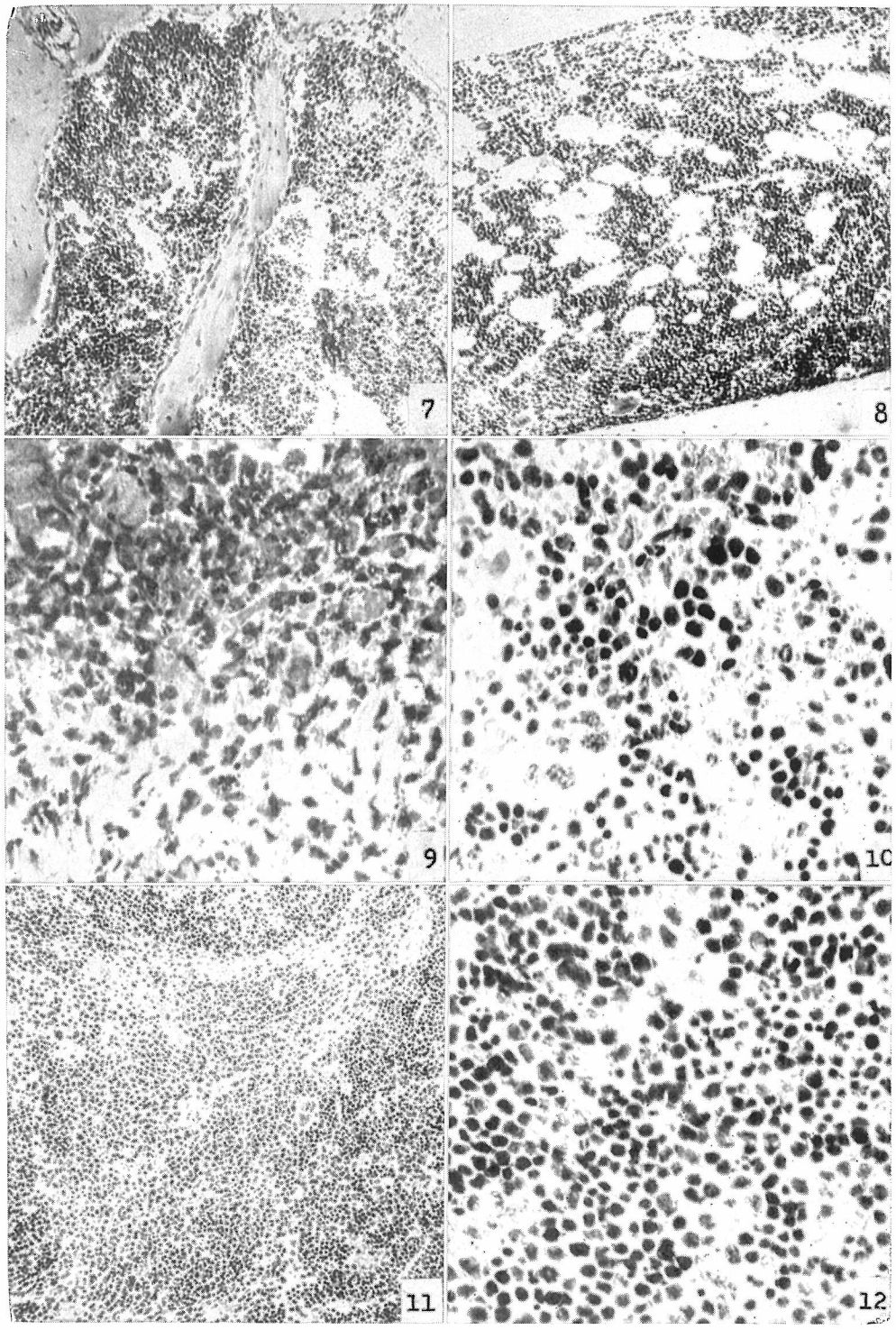
Fig. 21. Mesenteric lymphnode; 16 days after 900r gamma-irradiation and fresh IBM treatment. The lymphnode is completely wasted. H-E stain × 400

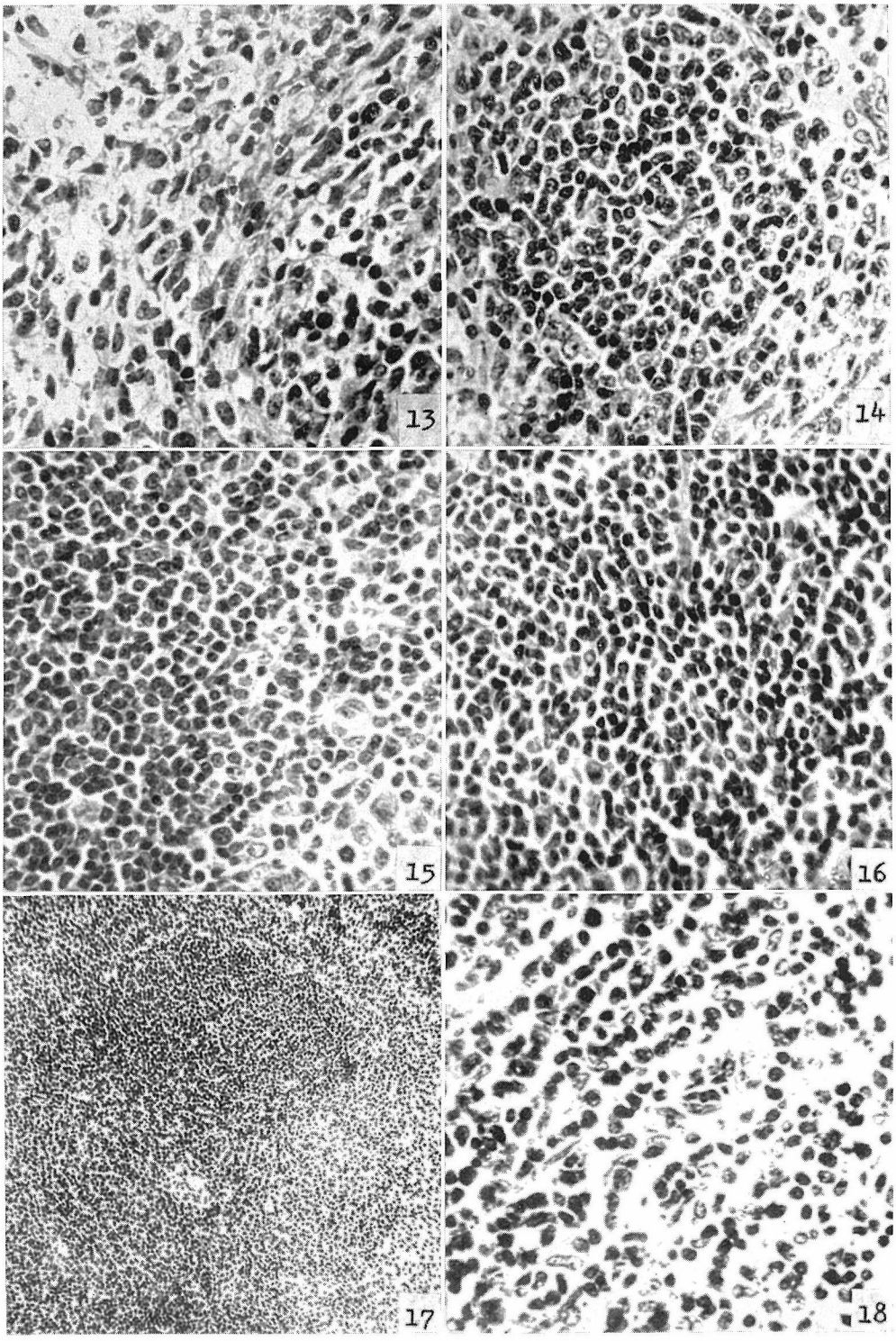
Fig. 22. Mesenteric lymphnode; 14 days after 900r gamma-irradiation and 360 days preserved IBM treatment. The lymphnode is completely wasted. H-E stain × 400

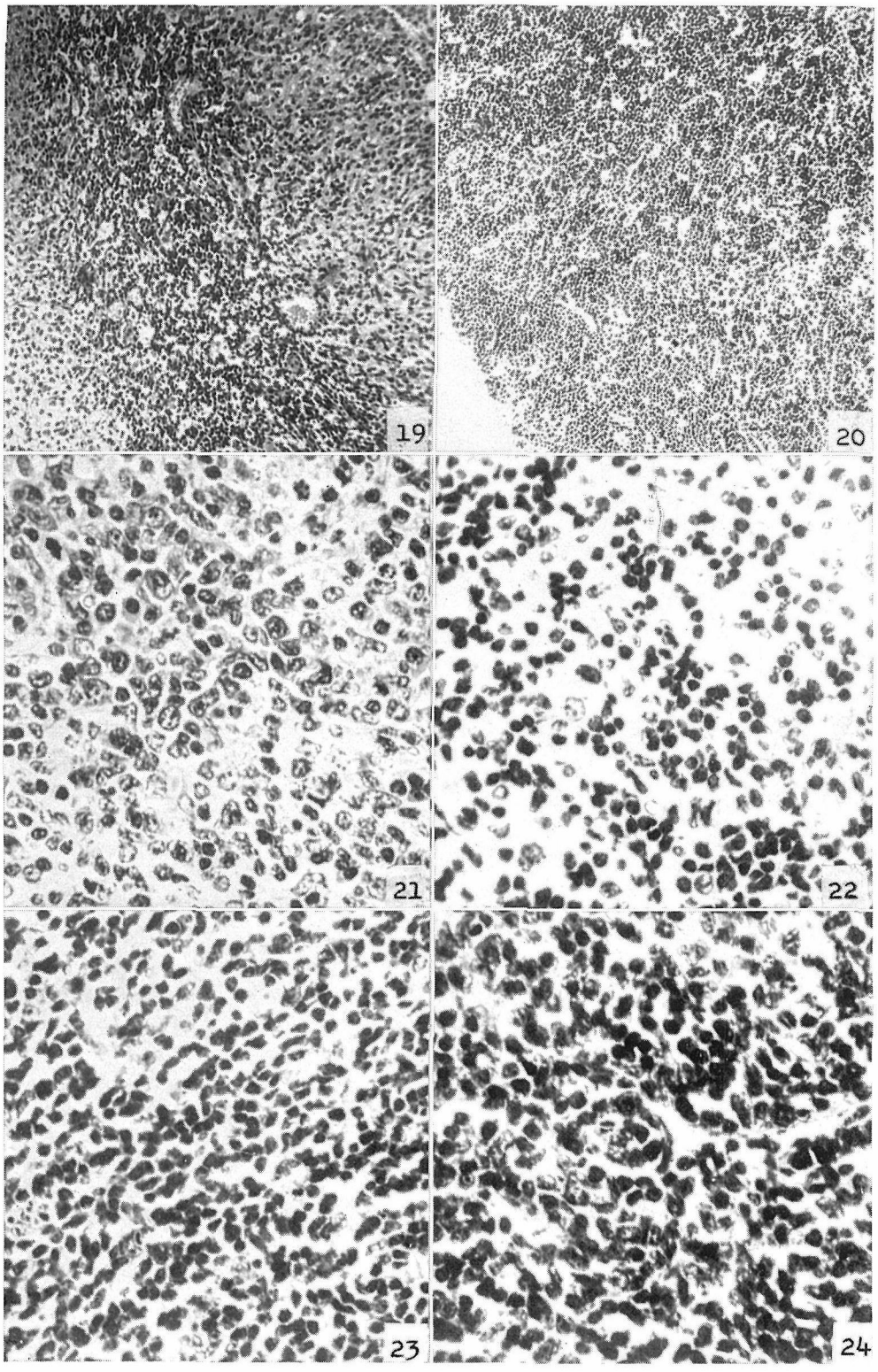
Fig. 23. Brachial lymphnode; 28 days after 900r gamma-irradiation and 450 days preserved IBM treatment. H-E stain × 400

Fig. 24. Mesenteric lymphnode; 30 days after 900 r gamma-irradiation and fresh IBM treatment. The cellularity is almost normal. H-E stain ×400


1
2
3
4
5
6


7
8
9
10
11
12


13
14
15
16
17
18


19
20
21
22
23
24